STUDIES IN EPIDEMIC (LETHARGIC) ENCEPHALITIS

CULTURAL STUDIES

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During the course of investigations carried on the past seventeen months, Strauss, Hirshfeld and Loewe,[1] and later Loewe, Hirshfeld and Strauss,[2] demonstrated that epidemic encephalitis was due to a filterable virus. This naturally suggested the application of methods for the cultivation of the filtrate virus. In a brief preliminary note [3] we described a filterable organism obtained from the virus with special cultural methods. On Feb. 11, 1920, before the New York Pathological Society, we presented in detail the experimental work on which this not was based.

In this communication we wish to review this work and to report additional studies carried out since that time.

LITERATURE

The literature concerning the etiology of this disease is contradictory. For the most part, the bacteriologic studies on blood, brain and cerebrospinal fluid have been negative. McIntosh[4] investigated cases of epidemic encephalitis with special reference to *B. botulinus* with entirely negative results. Recently he has succeeded in reproducing encephalitis in a monkey by injecting the Berkefeld filtrate of brain material from a fatal case.

Von Wiesner,[5] in Economo's Clinic, demonstrated a gram-positive diplostreptococcus in smears and cultures with which he claimed to have had positive results in animal inoculations.

Crookshank[6] expressed the belief that von Wiesner's organism was identical with the organism described by Rosenow[7] in 1916. The same organism was isolated from several of the English cases. Von Wiesner's work has received no further confirmation, the prevailing opinion being that he was dealing with a contamination.

Bradford, Bashford, and Wilson[8] report the finding of filtrable organisms in seven diseases, including epidemic encephalitis, trench fever and influenza. The bacteriologic studies were carried out with the aid of a modified Noguchi

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1 New York Med. Jour., 1919, 109, p. 772.
2 Jour. Infect. Dis., 1919, 25, p. 378.
3 Loewe and Strauss: Jour. Am. Med. Assn., 1919, 73, p. 1056.
4 Brit. Jour. Exper. Path., 1920, 1, p. 2.
5 Wien. klin. Wchnschr. 1917, 30, p. 933.
6 Lancet, 1918, 1, p. 653; 1918, 1, p. 699; 1919, 1, p. 79.
7 Jour. Am. Med. Assn., 1916, 67, p. 1202.
8 Quart. Jour. Med., Oxford, 1918, 12, p. 99, 9 pl.

tissue medium, using glycerolated brain and brain filtrate as inocula. The work on trench fever and influenza was questioned by Arkwright[9] following a study of Wilson's original cultures and smears. Both Bradford and Wilson in a note appended to Arkwright's article retract their claims as to having recovered in pure culture the agents of these two diseases. Their work on encephalitis is open to the same criticism, especially in view of inconclusive animal inoculations. However, we cannot help feeling that these investigators were proceeding along proper lines.

Brasher and others[10] found an organism in smears of the spinal fluid of two cases which was not the diplostreptococcus of von Wiesner, but a minute gram-positive coccus identical with that isolated by Bradford and others in cases of trench fever, influenza, and trench nephritis. Burnell[11] isolated from the blood in seven cases a gram-negative bacillus which was not motile and not sporeforming, and which he classified as belonging to the hemorrhagic septicemia group, or pasteurelloses. The author states that his findings are inconclusive, but warrant further investigation. Stafford[12] reports finding in smears of centrifugalized sediment of two spinal fluids, from the same patient, rather large, gram-positive diplococci which were cultivated with great difficulty on aerobic mediums only. Animal inoculations were negative.

Morse and Crump[13] report the isolation from the brains of six consecutive cases of lethargic encephalitis, a staphylococcus-like organism, resembling that described by Stafford. The organism was recovered in pure culture from the ventricular fluid. The organism is a gram-positive, non-motile coccus, morphologically like the staphylococcus. It is grown readily on the ordinary laboratory mediums. Their animal experiments were limited, being confined to the subdural inoculation of bouillon cultures into a few rabbits. Encephalitis was apparently produced, but detailed descriptions of the lesions are wanting. They concluded that the encephalitis is produced by a toxin generated by the growth of the organism and present in the filtrate of the cultures. No mention is made of an attempt to grow the filtered cultures on tissue ascitic fluid medium. In view of the readiness with which this organism was recovered, it is rather strange that it has not been found more universally.

Cleland and Campbell,[14] although obtaining negative bacteriologic findings, have stated definitely that in view of their positive animal inoculations, the causative organism is a filter passer with an affinity for the central nervous system. These investigators assert that they were not dealing with epidemic encephalitis, although some of their protocols would suggest that they were dealing with this disease.

Levaditi and Harvier[15] have confirmed the fact that epidemic encephalitis is due to a filtrable virus. They have reproduced our experiments in rabbits and monkeys, and in addition they have found that guinea-pigs are susceptible to this virus. Cultural experiments on the ordinary laboratory mediums were negative. They apparently did not make use of the anaerobic tissue ascitic fluid method.

[9] Brit. Med. Jour. 1919, 1, 233.

[10] Brasher, Caldwell and Coombe: Brit. Med. Jour., 1919, 1, p. 733.

[11] Med. Jour. Austral., 1917, 2, p. 157.

[12] Jour. Lab. and Clin. Med. 1919, 4, p. 11.

[13] Jour. Lab. and Clin. Med., 1920, 5, p. 5.

[14] Med. Jour. Australia, 1919, 1, p. 234.

[15] Comptes Rendus des Seances de la Soc. de Biologie, 1920, 83, p. 354; Soc. Med. des Hopitaux, 1920, pp. 179 and 583.

METHODS EMPLOYED

Our early negative cultural studies using ordinary laboratory mediums for aerobic and anaerobic culture, as well as the Rosenow technic, soon convinced us that we must be dealing with an organism of discriminating cultural requirements. This led us to the adoption of the tissue ascitic fluid medium introduced by Theobald Smith, developed by Noguchi, and so successfully used by him for the cultivation of Treponema pallidum and other organisms, and by Flexner and Noguchi in the growing the globoid bodies of poliomyelitis.

The original Noguchi technic has been followed carefully and is now being used exclusively as the organisms were evidently too highly parasitic to thrive on the modified mediums which suggested themselves. Plain, salt poor and dextrose broths were used as diluents, but with unsatisfactory results. Human, horse and rabbit serums, and inactivated ascitic fluid were all inimical to the growth of the organism. Due to the apparent resistance of monkeys to this disease it was not deemed wise to attempt the use of monkey serum. Solid and semisolid mediums proved unfavorable for the isolation of organisms directly from infectious material. These modifications were attempted for a two-fold purpose; first, to obtain a more luxuriant growth, and second, to conserve our supply of ascitic fluid. It was also found that the kidney tissue was essential.

The preparation of the medium as now used is briefly as follows:

Sterile kidney fragments are transferred to tubes 20 cm. x 1 1.2 cm., covered with 3 to 4 c.c. of sterile ascitic fluid, and incubated for 48 hours. At the end of this time the contaminated tubes are detected by gross examination and by dark field illumination, and discarded forthwith.

Successful cultivation depends in no small degree on the choice of a suitable ascitic fluid. We feel that we owe no small part of our success to the fact that our ascitic fluid had been stored over a relatively long period of time, and by virtue of its concentration was unusually rich in the elements necessary for the growth of the particular organism with which we were working. The ascitic fluid should conform to certain standards—it should be sterile, bile free, and of a high specific gravity. The presence of fibrin enhances the growth of the organism. Our most luxuriant cultures were obtained with ascitic fluid drawn from decompensated cardiac cases. Abdominal fluid from cases of cirrhosis of the liver can be used, providing

especial care has been taken to determine that traces of bile are not present. A negative blood Wassermann reaction had been obtained in all cases. Ascitic fluid secondary to peritoneal tuberculosis or carcinomatosis does not afford a suitable culture medium. In the final analysis the choice of an optimum ascitic fluid is an empirical one. It has been our custom to use several fluids in cultivating a given material.

There are no special precautions to be observed as regards the kidneys except that it must be removed in a sterile fashion. The kidneys may be extracted by the abdominal route or through lumbar incisions. Occasionally one finds an infected kidney, even after observing the strictest asepsis. Sterile testes from rabbits can also be used. As pointed out by others, the kidney serves a dual purpose—first, to afford special form of nutrient and second, to create a more perfect anaerobiosis.

The tubes containing sterile kidney tissue and ascitic fluid are inoculated, and ascitic fluid added to form a column about 10 cm. high. Petrolatum of low melting point is heated and poured over the surface of the fluid in a layer about 1 cm. thick. The petrolatum quickly cools and effectually seals the tube, producing almost perfect anaerobic conditions. In the beginning the cultures were layered with sterile liquid albolene, which necessitated the use of Novy jars. The use of petrolatum instead of liquid albolene has rendered unnecessary the use of the Novy jars. The petrolatum used in our work is autoclaved in small Erlenmeyer flasks for one hour at 15 lbs pressure. A number of tubes are always inoculated with a given material and usually with different amounts. As the frequent opening of the tubes during the preparation of the medium offers many opportunities for contamination, it is necessary to use the utmost care during the whole process, and it is only by the use of numerous controls that false results can be guarded against. Control tubes are set up as follows: (1) serum and petrolatum, (2) inoculum, serum and petrolatum, (3) serum, kidney and petrolatum.

Both controls and inoculated tubes are incubated at 37 C.

The optimum solid medium is of a gelatinous consistency, made so by the addition of 1 part of 2% nutrient agar to 4 or 5 parts of ascitic fluid, the kidney tissue being added as usual. The customary controls are also made.

The following method of examining and subcultivating solid cultures was suggested to us by Dr. Noguchi: The cotton stopper is first paraffined. A diamond pencil is drawn horizontally across the test tube just below the region to be subcultivated. The tube is immersed, not longer than a minute, in a hot solution containing equal parts of 95% alcohol and saturated bichlorid of mercury. A heated glass rod is applied to the center of the scratch mark made by the diamond pencil. This causes the tube to crack sharply around its entire circumference. The ends of the tube are then separated for a distance of 1 cm. and the agar column gently broken at the desired place. The exposed agar of the upper fragment is dipped in the alcohol-bichlorid solution. The portion of the agar which has become clouded is scraped away with a platinum spatula. By pressing on the paraffined stopper as much agar is extruded as is desired. The tube is then inverted and placed in a shallow receptacle. Single colonies are picked with sterile capillary pipets, if necessary, with the aid of a magnifying glass. The pipets containing the single colonies are washed in tubes of fluid medium, which are then treated in the usual fashion. Smears are made by crushing between two slides bits of agar containing colonies.

MATERIAL CULTURED

The materials cultivated were:

1. Fragments, salt solution emulsions and Berkefeld filtrates of salt solution emulsions of brains of patients and of inoculated animals. It has been our aim whenever possible to use the fragments, since they require little manipulation, and for other reasons to be mentioned. This has been possible in only a small percentage of human brains, and a somewhat larger percentage of brains of animals, due both to the presence of antemortem invaders, and to contamination in the process of removal of the brains. Blocks of brain were taken preferably from the midbrain where the most pronounced lesions of this disease are found. Material for inoculation was removed with the strictest sterile precautions and with the brain in situ. Berkefeld filtrates were used in practically all instances, especially when there was the slightest possibility of contamination. The filtrates were prepared from 5% salt solution emulsions made by grinding in a sterile mortar with sterile sand. Uncontaminated emulsions were also cultivated. When it is desirable to inoculate a large amount of the original filtrate or

emulsion, we have found it of service to concentrate these in vacuo at 36 C. Grossly contaminated material has been stored in the refrigerator in 50% glycerol, and subsequently cultivated en bloc, or ground up and filtered.

2. Cerebrospinal fluid, removed under sterile precautions, from patients and animals, was cultivated in amounts from 0.5 to 1.0 c.c.

3. Blood was drawn under aseptic precautions.

4. Material from the nasopharynx was obtained from living patients by means of nasal irrigations, pharyngeal swabs, or the West tube. At necropsy nasal mucous membrane and nasopharyngeal mucous membrane were removed by means of the curet. In animals, the whole nasopharyngeal mucous membrane was dissected out. Nasal washings were filtered directly, unless the presence of an excessive amount of mucus made it necessary to shake the washings with glass beads. Pharyngeal swabs were washed in several changes of salt, taking care to use small quantities. The slightly turbid fluid so obtained was then filtered. Nasopharyngeal mucous membrane was finely emulsified by grinding in a mortar with sand and salt solution, and then submitted to filtration.

The filters used in the beginning of the work were the standard Berkefeld 5N or W, but lately, due to difficulty in obtaining Berkefeld filters, we have been using the Mandler filters, which we have found satisfactory as regards their ability to hold back the usual test organisms. As tested by the manufacturer, they will retain 8 to 12 lbs. of air pressure without passing an air bubble, after having been immersed in water 12 hours. As tested by us, they hold back B. prodigiosus under the same conditions obtaining in our experimental filtrations. All filtrates before being used were cultivated on ordinary laboratory mediums to insure sterility.

Controls.—Control studies were carried out on nasal washings, nasopharyngeal mucous membrane, blood, and cerebrospinal fluids of patients suffering from, or dead of, diseases other than lethargic encephalitis. Patients from the surgical wards were preferred so as to minimize the possibility of contact with the disease under investigation. The nasopharyngeal mucous membrane and brains of normal rabbits were also studied. Brains from patients dying of various diseases were used as controls. Spinal fluid from healthy monkeys was used to control the cultural findings.

GROSS APPEARANCE OF THE CULTURES

In spite of the numerous precautions used in the preparation of the culture tubes, a small percentage of contaminations invariably appear. These are for the most part due to the numerous manipulations necessary before sealing the tubes. A small number apparently only make their appearance when all three elements—inoculum, kidney and ascitic fluid—are combined. Most contaminations are readily detected grossly, by the dense clouding of the tube, gas formation, evidence of putrefaction, and the rapid disintegration of the kidney. Other contaminations make their appearance on the aerobic subcultures on ordinary laboratory mediums, which are made whenever a tube is exposed. The gross findings are confirmed by dark field illumination and by examination of smears stained by Gram's method. Generally gross contaminations are detected within a few days after the tubes have been set up.

A successful growth is usually manifested on the fifth to the seventh day by clouding of the medium commencing about the kidney tissue, the outline of which becomes hazy and irregular. The clouding extends upward rapidly to within about 1 cm. of the top of the ascitic fluid column. In a few instances in which blood from the kidney has diffused into the body of the culture, it is gradually decolorized in the presence of a positive culture. The organisms are not held in suspension long, but tend to form clumps which settle to the bottom of the tube, leaving the supernatant fluid clear. The degree of clouding is no criterion of the activity or presence of growth, for occasionally one is surprised to find in smears from relatively clear tubes, numerous organisms. The clouding of the medium is due partly to the growth of the organism itself, and partly to protein precipitation by the acid producted by the organisms. This is especially marked in solid cultures on dextrose serum agar, surrounding the individual colonies.

Transfer to solid mediums can be obtained only with the later generations of the organism. In no instance were we able to grow the organism on solid mediums directly from infectious material. The growth in solid mediums assumes different forms, depending on the adaptibility of the given strain to this type of culture medium. When the organisms are numerous, a diffuse clouding of the medium is observed. Individual colonies can only be made out with a magnifying glass. The initial and most intense clouding takes place in the region

of the kidney and extends upward. When the organisms are few in number, minute colonies may appear only in the region of the kidney, or occasionally scattered throughout the medium. The colonies gradually increase in size so as to become easily recognizable with the naked eye.

MICROSCOPIC STUDY OF THE ORGANISM; MORPHOLOGY

Studies of the fluid cultures under dark field illumination reveal the organisms as minute globular refractile forms, occurring singly, in diploform, chains and clumps, the latter form predominating, especially in the older cultures. These bodies show active Brownian motion, but no true motility. When motile forms are found in the dark field, the culture is at once discarded as contaminated.

In stained smears the organisms appear as minute globular bodies which are arranged singly, in diploform, in chains, and clusters. The chain formation is most marked on solid mediums, due to the fact that they are unfavorable for their growth. This observation is analogous to the Pfaundler phenomenon, according to which typhoid bacilli grown in immune serum tend to form chains. The organism has an average diameter of 0.25 mikrons as measured by the ocular mikrometer. Smaller forms are found in young cultures, and larger more deeply staining degenerated forms are seen in the older cultures. The reaction to Gram's stain depends a great deal on the medium used and on the age of the individual culture. Young cultures and those grown on fluid mediums are mostly gram-positive, while the older cultures and those grown on solid mediums tend at times to be gram-negative. In the early work reliance was placed on Loeffler's alkaline methylene blue for want of a satisfactory Giemsa stain. Subsequently several other stains have been tried out which have proved valuable in establishing a definite tinctorial reaction for the organism, which is of basophilic nature.

STAINING METHODS

Loeffler's Alkaline Methylene Blue: Stain from one to two hours; preparations fixed only in methyl or absolute alcohol. The organisms stain a violet hue, which differentiates them from the blue background. The disadvantage with this method is the densely staining background containing tissue particles, which tend to obscure the field. This method has been used for routine rapid work.

Giemsa solution may be used in two ways: the rapid, and the overnight method. It is only lately that we have been able to secure a satisfactory Giemsa stain. The smears are previously fixed in absolute alcohol for ½ to 1 hour, followed by ether for 2 or 3 minutes. The slides are then immersed in dilute Giemsa 1-10 or 15) and permitted to stain overnight. With a more concentrated solution smears can be stained in ½ hour with the aid of heat, care being taken to heat the slides only to steaming. Giemsa smears are more satisfactory for studying the morphology of the organisms.

Unna's Alkaline Methylene Blue: Stain for 1 hour, after fixation in methyl or absolute alcohol for ½ to 1 hour. Differentiation is accomplished by means of a glycerol-ether solution (equal parts of glycerol-ether, 1 part, to 4 parts distilled water). We have obtained a selective stain by this method whereby the organisms stand out as dense purplish bodies on a clear background.

Ljubinsky's Pyoktannin-Acetic Acid: Stain for 45 minutes, after fixation in methyl or absolute alcohol for ½ to 1 hour. Care must be taken in the use of this method since most of the preparation is lost through the solvent action of the acetic acid. The organisms appear dark blue to black.

Unna-Pappenheim: Stain for 1 to 1½ hours, after fixation in absolute alcohol or equal parts of absolute alcohol and saturated mercuric chlorid. This stain serves best for impression smears of brain material. The organisms stand out red against the green of the surrounding tissue.

Pyronin, 1% Aqueous solution: For prolonged staining (overnight). The organisms stain red.

We have occasionally experienced difficulty with precipitate and sediment in these stains. When this occurred the precipitate was removed by centrifugalization, filtration, or, if necessary, by filtration through a Berkefeld candle. We wish to emphasize that heat was never used in the fixation of smears.

RESULTS OF CULTURAL STUDIES

Human Nasopharyngeal Membrane Recovered at Necropsy.—The organism has been recovered in pure culture from Berkefeld filtrates of nasopharyngeal mucous membrane of 7 fatal human cases of epidemic encephalitis. In 10 attempts we have been successful in 7. One

of these strains has been carried to the sixteenth generation and another to the twelfth generation, in artificial cultures, without animal passage. The later strains proved pathogenic for animals.

Controls of filtrates of nasopharyngeal mucous membranes of patients dying of other conditions (cardiovascular disease, megacolon, peritonitis, mediastinal tumor, carcinoma of the stomach, post-operative hemorhhage and empyema) have all been sterile.

Human Nasopharyngeal Washings.—Filtrates of nasopharyngeal washings from 23 cases of epidemic encephalitis were cultivated with

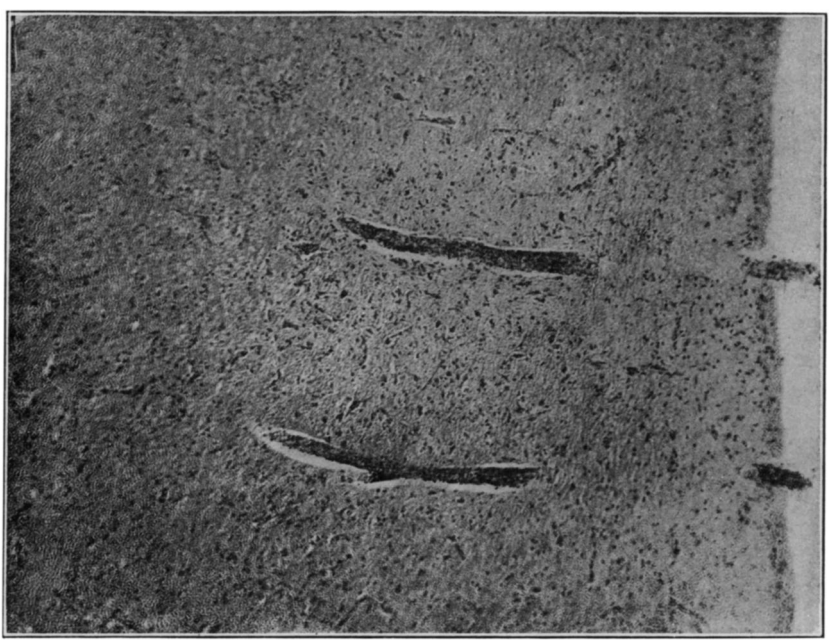

Fig. 1.—Monkey brain, section of pons showing perivascular (adventitial) infiltration with mononuclear cells. Animal injected intracranially with culture originally derived from Berkefeld filtrate of human nasopharyngeal mucous membrane removed at necropsy.

positive findings in 15 cases or 66%. Many of these strains were subcultured successfully and carried along through several generations. The organism was recovered from brains of rabbits injected with the virus of those nasal washings in 8 instances, as well as from the brains of rabbits injected with the organisms from these nasal washings in 5 instances.

Control studies of nasal washings were negative in 8 cases (mastoiditis (2), sinusitis, pyelitis, appendicitis, empyema, cholelithiasis and nephrolithiasis).

Rabbit Nasopharyngeal Mucous Membrane.—The organism was recovered from the nasopharyngeal mucous membrane of 3 rabbits injected with (1) human mucous membrane filtrate, (2) sixth generation of virus from a human mucous membrane, (3) culture from human mucous membrane in sixth generation. Those cultures have been carried, one to the sixth, and the others to the third generation.

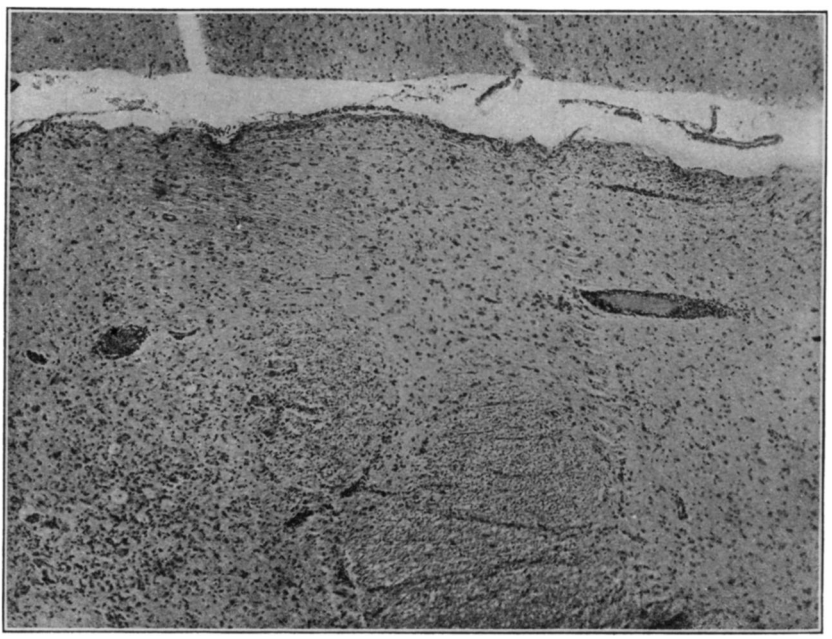

Fig. 2.—Monkey brain; collars of round cells about vessels near floor of fourth ventricle. Animal inoculated intracranially with culture derived from Berkefeld filtrate of human nasopharyngeal mucous membrane removed at necropsy.

The organism was recovered in 2 instances from the brains of animals injected with the Berkefeld filtrate of these rabbit mucous membranes, and in 2 instances from the brains of rabbits injected with the organism derived from these rabbit mucous membranes.

Cultures of Berkefeld filtrate of nasopharyngeal mucous membrane from 7 normal rabbits were used to control these cultures and gave negative results.

Cerebrospinal Fluids.—Cerebrospinal fluids have yielded the organism in 12 out of 24 cases. It was found in one case on direct smear of the sediment of the centrifugalized spinal fluid. These strains were carried in one instance as far as the eighth generation; in another as far as the fourth; but for the most part only through the second generation, as this was deemed sufficient to prove the viability of the culture. The organism was recovered from the brains of 8 rabbits injected with the spinal fluid itself, and from the brains of 4 rabbits injected with the organism derived from these spinal fluids.

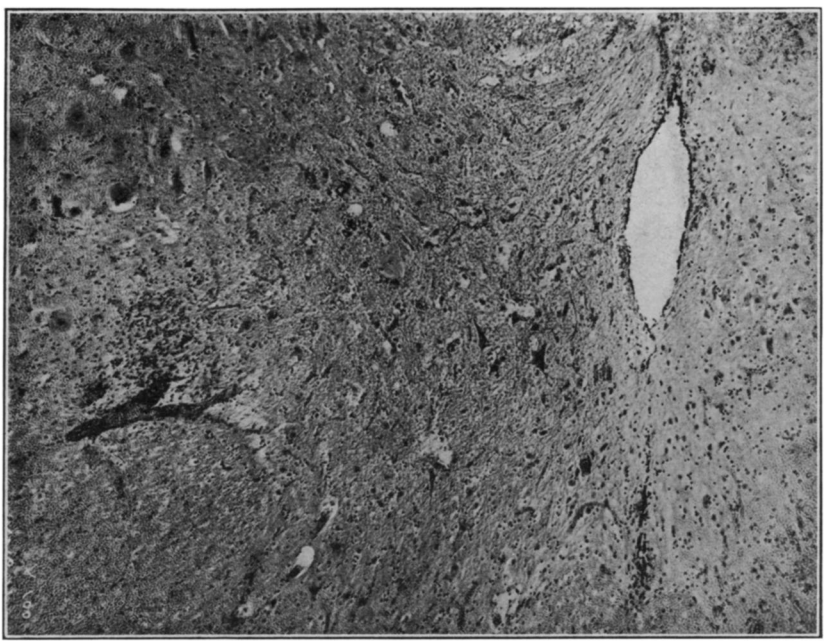

Fig. 3.—Rabbit cord; section of upper dorsal cord. Focus of round cells in proximity to vessel showing adventitial infiltration with mononuclear cells. Animal inoculated intracranially with culture obtained from Berkefeld filtrate of human nasopharyngeal mucous membrane removed at necropsy.

Eight spinal fluids from patients suffering from various diseases than epidemic encephalitis (brain abcess, brain tumor, psychasthenia, uremia, multiple sclerosis, tuberculous meningitis, neurosyphilis, and spinal cord tumor) were cultivated with entirely negative results. Animal inoculations were likewise entirely negative in 6 cases. Cultures and animal inoculations of spinal fluid were made in most cases immediately after withdrawal of the fluid.

Rabbit Brains.—A total of 56 rabbit brains were cultivated, using Berkefeld filtrates of brain, blocks of brains and emulsions of brain; there were 36 positive results, 64%. Ten emulsions were inoculated with 3 positive results. Eleven blocks of brain were inoculated with 10 positive results. The relatively small number of blocks and emulsions cultivated is due to the difficulty in removing the material in sterile fashion. In 3 instances, blocks of brain gave positive results when filtrates did not. This givest a total successful recovery of the organism from 39 of 56 rabbit brains cultured, 69%. There was one

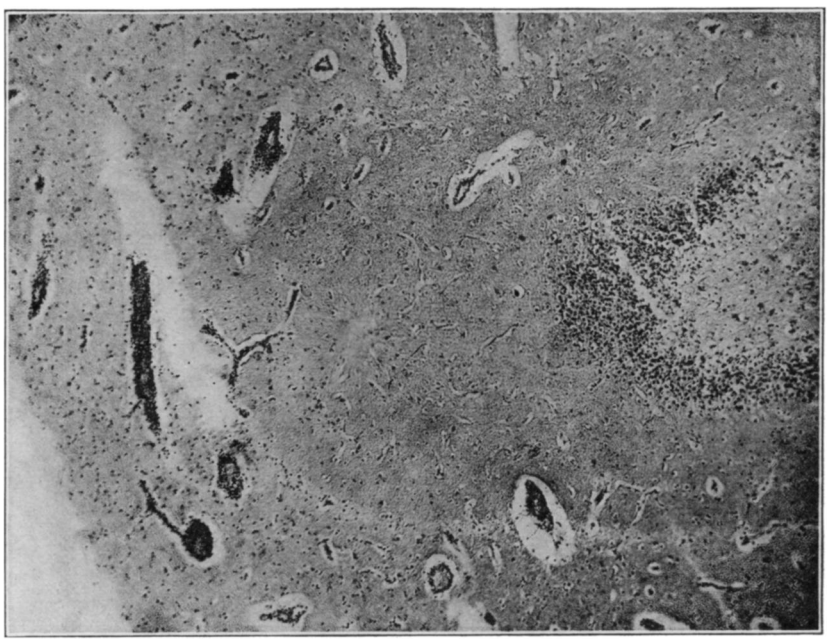

Fig. 4.—Rabbit brain; area showing mononuclear infiltration of adventitia (Virchow-Robins space) of blood vessels in subcortex. Animal inoculated intracranially with culture of cerebrospinal fluid from human case.

series of 7 animal transmission initiated by a Berkefeld filtrate of a human nasopharyngeal mucous membrane. The organism was recovered following each transmission. One of the organisms recovered from this series was injected into animals in the fourth, fifth, sixth, seventh and eighth generations, and was recultivated from 50% of the brains so inoculated. Rabbits were injected with the fourth, fifth, sixth, eighth and eleventh generations of a culture from a human

nasopharyngeal mucous membrane and the organism was recovered from the brains of 4 of these animals, or 50%. A 3 months old glycerolated filtrate from human nasopharyngeal mucous membrane was injected into animals and carried through two transmissions, and the organism recovered from 50% of the rabbit brains. The same

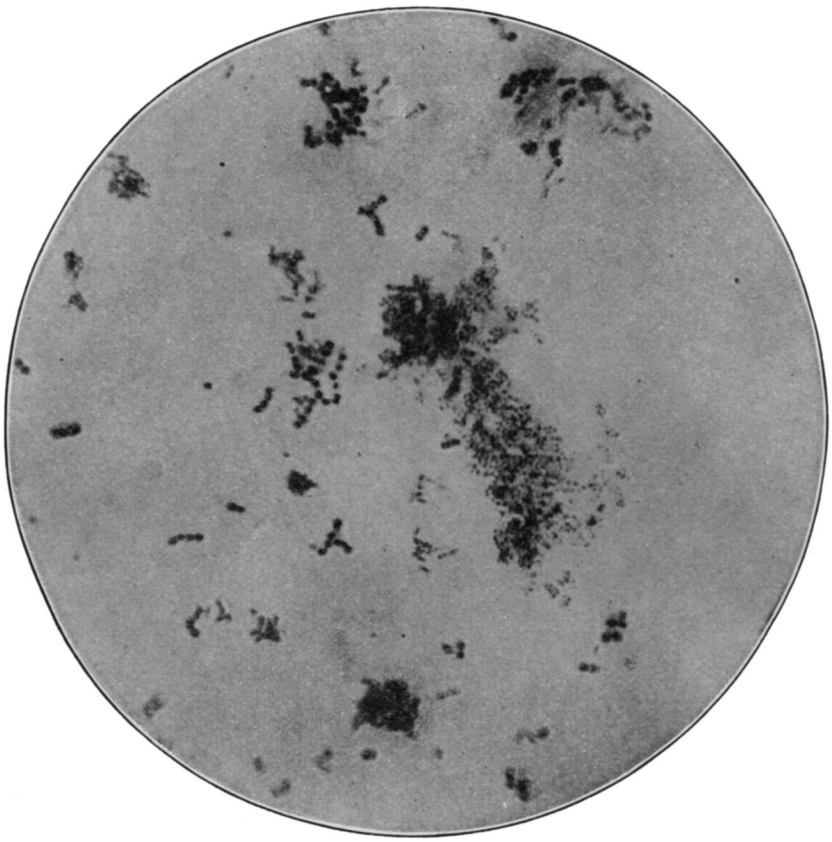

Fig. 5.—Seven day old fluid culture; superimposed anhemolytic streptococcus for comparison; × 1200.

filtrate when 4 months old was injected into 2 animals, with recovery of the organism from both.

In a series of transmissions initiated by spinal fluid from a case of encephalitis, the organism was recovered from the brain in each of the transmissions, the positive results making a total of 5 of the 8

brains cultivated. The second generation of one of the recovered organisms was injected into animals. Cultures of the brains of these animals were positive.

Monkey Brains.—Cultures were made of 6 monkey brains injected with virus of various kinds (filtrates of human nasopharyngeal mucous membrane, and of infected nervous tissue from rabbit, monkey and

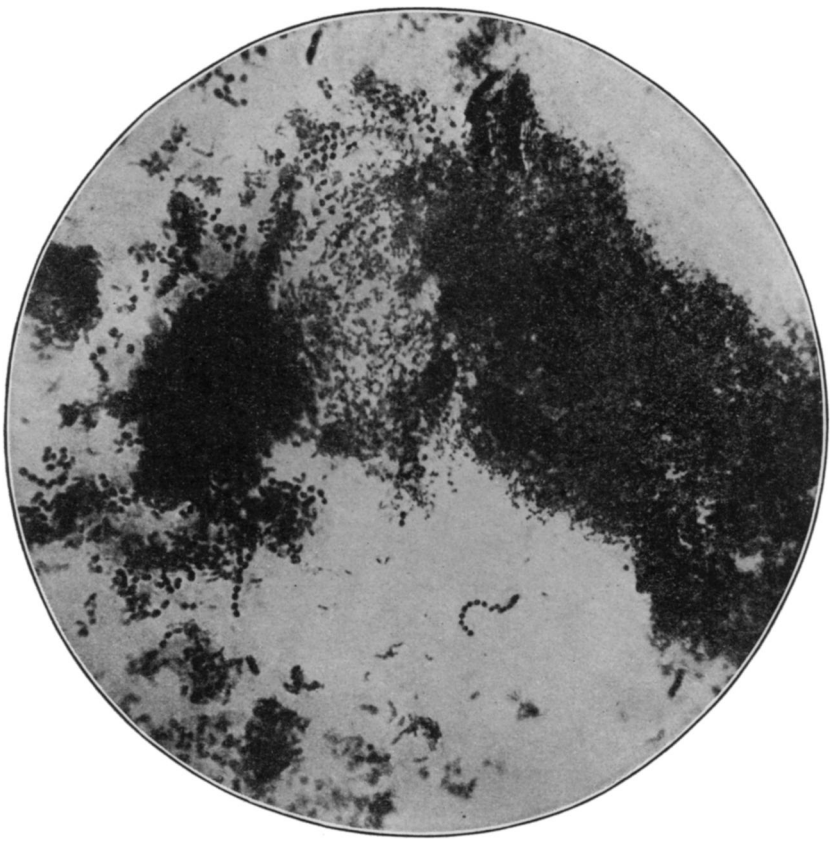

Fig. 6.—Fluid culture 3 weeks old; superimposed anhemolytic streptococcus for comparison; × 1200.

human) and 5 positive results were obtained. The organism was demonstrated in cultures of blocks of a glycerolated brain kept in the refrigerator for 3½ months. It was isolated from the brain of a monkey which developed a secondary B. subtilis infection after a subdural inoculation with the Noguchi culture of a human naso-

pharyngeal mucous membrane. The presence of the B. subtilis was not detrimental to the organism. A positive culture was obtained from the brain of a monkey that was injected with the seventh generation of a Noguchi culture of human nasopharyngeal mucous membrane.

Human Brains.—After numerous attempts and by various means, we have finally been able to isolate the organism from human brains. We achieved our earliest and best results with a case of lethargic encephalitis that ran a rapid course, death ensuing in 5 days. Positive cultures were obtained on the first attempt by the use of large blocks of tissue. Subcultures of this organism proved pathogenic for rabbits. In another case, we were able to use only filtrates because the brain was grossly contaminated. This probably accounts for the great difficulty experienced in isolating the organism. In a third instance, an emulsion of a human brain was successfully cultivated. The original emulsion produced the typical clinical and pathologic pictures in the monkey. Our results have shown that the organism is more readily recovered from the brains of those cases that run a rapid course. Occasionally we have resorted to concentration in vacuo in order to obtain successful growths.

Blood.—Cultural studies of the blood were carried out in two cases and in both the organism was recovered. Both of these cases were of the wild maniacal type with myoclonus. In one case the infection was so overwhelming that death ensued within six days.

The tubes were inoculated at the bedside without the use of anticoagulants. In one case positive cultures were also obtained by the use of a large quantity of blood laked with sterile distilled water. The organisms were separated from fragmented blood cells by repeated subcultures.

PATHOGENICITY OF CULTURES

As in the case of the virus, monkeys are apparently refractory to cultures of the organism. The two following protocols are of interest:

Monkey 17 was injected subdurally with 2 c c of Berkefeld filtrate of the culture of human nasopharyngeal mucous membrane. The culture was of the tenth generation; the individual culture being 3 weeks old. Complete paralysis of both hind legs was first noted 10 weeks after the operation. This gradually spread to involve the right foreleg. The spinal fluid contained 68 cells per cmm. Sections of this brain show what are apparently old, healing lesions in the midbrain. There is considerable proliferation of the adventitia cells of the blood vessels. Monkey 20 was injected intracerebrally with 2 c c of a Berkefeld filtrate of the culture of another human nasopharyngeal mucous membrane.

This culture was originally derived from a 50 per cent. glycerated filtrate kept on ice for 3 months, and had been carried through 7 generations. This monkey was well for 3 weeks, and then developed elevation of temperature and apathy. There was profound lethargy and progressive paralysis of all four extremities. Death ensued within 5 days. The pathologic picture was typical of encephalitis. The organism was recovered from the brain.

We have found that the incubation period is increased by prolonged cultivation on artificial medium and also by filtration of the culture, which results in the removal of a considerable number of the organisms.

Our rabbit inoculation experiments have been carried out with a number of strains preferably in the later generations. About 50% have succumbed with typical lesions. The incubation period varies from 2 to 42 days. In view of this apparent natural immunity a series of rabbits were always inoculated, reliance never being placed on inoculation of a single rabbit.

Three strains, derived from filtrates of nasopharyngeal mucous membrane from lethargic encephalitis cases, have been used. Filtrates of cultures from the third to the eleventh generations were injected intracranially into 20 rabbits. Twelve succumbed with typical lesions. Further successful animal transmissions were made with filtrates of these brains. One animal injected intracranially with 0.2 c c of a filtrate of a 6 weeks old culture in the fourth generation, first showed paralysis of both hind legs 5 weeks after inoculation. The paralysis slowly progressed to involve all four extremities. Typical pathologic lesions were present both in the brain and in the spinal cord. One strain partook of the characters of the virus from which it was isolated, in that it tended to produce hemorrhagic lesions.

Colonies picked from solid cultures of these strains have been grown in fluid medium. These cultures when injected intracranially into rabbits have produced lesions in 5 of 8 animals so inoculated. Cultures derived from brains of inoculated animals proved pathogenic for rabbits in approximately half of the animals injected.

Cultures of organisms from cerebrospinal fluids and of organisms isolated from the brains of rabbits injected have produced lesions in 5 of 12 animals inoculated. The infectivity of cerebrospinal fluid seems to be proportionate to the increase in cells.

We wish to bring out in connection with our animal experiments that we have not only produced typical lesions in rabbits with cultures derived from virus of various kinds, but we have also been able in many instances to recover the organism from the brains of animals

so injected and also to produce again the disease in animals with later generations of these same organisms. We were unable to produce lesions in animals injected in the same manner with control cultures.

DISCUSSION

The experimental evidence that has been presented, would indicate an etiologic relation of the filterable organism that we have isolated, to the disease in question. One possible objection that may be raised is that our successful animal inoculations with cultures have been due to carrying over of sufficient original virus to produce the disease. In our method of making transplants, only 0.2 c c of living cultures are used. It can thus be seen that in later generations the amount of virus carried over from the initial culture through the several subcultures is infinitesimal as compared with the amount of virus required originally for successful animal inoculations. Since the time elapsing between a first and eleventh generation is a matter of months, and in view of the rapidity with which virus deteriorates at incubator temperature, it is evident that original virus cannot be responsible for the potency of our cultures. Further, while the cultures lose in virulence through artificial cultivation over a long period, the incubation period in animals is not greatly prolonged. The symptoms and lesions produced by later generations are just as typical as with earlier generation and with virus. With this point in mind the following experiment was made: Two-tenths c c of a virulent culture was inoculated into a tube of tissue ascitic fluid medium, 15 c c in volume; 0.2 c c of the resulting mixture was then transferred to a similar amount of the same medium and so on for 6 dilutions. One c c taken from the fourth and sixth dilutions were inoculated intracranially into each of 6 rabbits, with entirely negative results. It has already been shown that by using solid cultures, we were able to pick single colonies and to grow the organism in pure culture in this way, and to inoculate successfully animals with the cultures so obtained.

The organism isolated resembles in morphology and cultural characteristics that found by Flexner and Noguchi in poliomyelitis. It differs in virulence, occurrence, and particularly in the ability to infect rabbits. Successful inoculations in the monkey and the rabbit have been obtained with the later generation of our organism. The isolation of organisms from spinal fluid sharply differentiates this disease from poliomyelitis. It is of interest that Foster [15] has isolated a fil-

trable organism from common colds which in morphology and growth resemble the globoid bodies of Flexner and Noguchi, and the organism which we have found in epidemic encephalitis.

It is evident that a new field in bacteriology has been opened up by the investigations of Noguchi. We believe that further investigation will prove that there is a group of filtrable organisms, resembling each other in morphology, but possessing distinctly different pathogenic characteristics. New methods must be introduced for the separation of the organisms of this group.

SUMMARY AND CONCLUSIONS

In our reported investigations we have brought forward this evidence:

Berkefeld filtrates of brain material, nasopharyngeal mucous membrane and nasal washings from cases of epidemic encephalitis have produced in rabbits and monkeys lesions typical of this disease. Spinal fluid and blood have also produced the disease experimentally in these animals. Many of these animals have succumbed with the typical picture of epidemic encephalitis. The virus has been passed through many series of animals. It can be preserved for many months in 50% glycerol.

Cultures made on ordinary mediums and by Rosenow's technic have proved negative.

By means of the ascitic-tissue culture methods perfected by Noguchi, we have been able to cultivate a minute, filtrable organism from cases of epidemic encephalitis: brain, nasopharyngeal mucous membrane, nasopharyngeal washings, spinal fluid and blood.

This same organism has been recovered from the brain and nasopharyngeal mucous membrane of animals that have been inoculated with virus and cultures and which have succumbed to the experimental disease. The cultures thus recovered from these animals have produced the disease when injected into other animals and the organism has again been recovered. Positive animal inoculations have been obtained with the eleventh generation of this organism.

Isolated colonies of the organism grown on solid Noguchi medium have been picked and pure fluid cultures secured. These fluid cultures have also produced encephalitis in animals.

Our results indicate that epidemic encephalitis can be differentiated from epidemic poliomyelitis for these reasons: Rabbits are susceptible

to infectious material from epidemic encephalitis and not from poliomyelitis. Monkeys are very susceptible to poliomyelitis and relatively refractory to material from epidemic encephalitis. Spinal fluid from poliomyelitis is innocuous when injected into rabbits and monkeys, whereas spinal fluid from cases of epidemic encephalitis produces in both of these animals lesions typical of the disease.

Control studies have been uniformly negative with material obtained from human patients suffering from or dead of, conditions other than epidemic encephalitis.